

Full Length Research Paper

Effect of phosphorus limiting on phytase activity, proton efflux and oxygen consumption by nodulated-roots of common bean (*Phaseolus vulgaris*)

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This work intended to measure the nodulated-roots oxygen consumption, proton efflux and phytase activity in 2 lines of common bean (*Phaseolus vulgaris*) (115, 147) at 2 levels of P supply. Rooted seedlings were inoculated with *Rhizobium tropici* CIAT 899 in hydroaerobic cultivation under glasshouse. Phosphorus was supplied as KH_2PO_4 at 15 and 250 $\mu\text{mol pl}^{-1} \text{ week}^{-1}$ (15P and 250P, respectively). Our results showed that plant growth nodulation and symbiotic nitrogen fixation were significantly affected by P limiting (15P) for the both lines, but this adverse effect was more pronounced in 147 than in 115. For the both lines, the phytase activity, higher in roots than in nodules, was significantly increased by P limiting, but 115 maintained higher values as compared to 147 line. In cotyledons, the phytase activity was higher in 115 than in 147. Phosphorus shortage increased the cumulated proton release only in 115, whereas it was lowered for 147. In this line, the proton release was linked to symbiotic nitrogen fixation. Under 15P, the proton efflux per unit of nodulated-root biomass was 25% greater for 115 than 147, suggesting that under P limitation, proton efflux may constitute an efficient way to increase P uptake in the tolerant line (115). 15P increased significantly nodulated-root O_2 consumption per g nodule DW and nodule conductance, but to a higher extent in 147. As a whole, bean plants at P-deficient conditions increased the activity of phytases and proton efflux, thus maintaining the oxygen diffusion in nodules. This may represent an adaptive mechanism for N_2 -fixing legumes to respond to P deficiency, by increasing the utilisation and the uptake of phosphorus for symbiotic nitrogen fixation.

Key words: bean, phosphorus, phytase activity, proton efflux, nodulated root oxygen consumption, phosphorus use efficiency for symbiotic nitrogen fixation.

INTRODUCTION

The availability of P is one of the most significant determinants of plant growth (Wang et al., 1998). Plants depend almost exclusively on P absorbed from soil. Total P in soil is abundant, but is rarely available (Liu et al., 1994). Phosphorus deficiency has a negative impact on biological N_2 fixation, since the dinitrogen fixation as well as the

ammonium assimilation into amino acids and ureides that occur respectively in bacteroids and in the plant cell fraction of nodules are energy consuming processes depending on the energy status of the nodules (Sa and Israel, 1991). The impaired biological N_2 fixation in P-deficient plants is usually explained by an effect of the low P supply on the growth of the host plant, on the growth and functioning of the nodule, or on the growth of both the plant and the nodule (Almeida et al., 2000). In common bean, soybean, lupin and alfalfa, P deficiency has been shown to reduce the number and biomass of nodules

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as well as their nitrogenase activity (Kouas et al., 2005; Qiao et al., 2007). In addition, phosphorus deficiency has previously been reported to decrease nodule mass more than host growth in soybean and common bean (Drevon and Hartwig, 1997; Kouas et al., 2005). Concerning nodule functioning, Ribet and Drevon (1995) reported that P deficiency increases the nodule conductance to O₂ that is postulated to regulate the O₂-limited nitrogenase activity (Drevon et al., 1988; Drevon and Hartwig, 1997).

Large differences have been reported for the biological dinitrogen fixation of various legume species and cultivars. According to Minchin et al. (1985), Hunt and Layzell (1993) and Witty and Minchin (1998), the variable oxygen barrier in the outer cortex of legume nodules regulates the diffusion of oxygen into the infected tissues and thus controls respiration and dinitrogen fixation. Some particular strategies have been observed for the adaptation of nodulated leguminous plants to limited P supply, such as the maintenance of concentrations of P in nodules much higher than in other organs (Pereira and Bliss, 1987), higher absorption of P from the solution directly by the nodules and bacteroids (Al-Niemi et al., 1998), increased N₂ fixation per unit of nodule mass to compensate for reduced nodulation (Almeida et al., 2000), higher accumulation of soluble sugars in nodules than in roots and shoots (Olivera et al., 2004) and higher O₂ consumption per unit of reduced N₂ associated with a higher nodule permeability (Schulze and Drevon, 2005).

It is well documented that plant genotypes differ greatly in their adaptive mechanisms to P deficiency. To improve growth under P-deficient conditions, P-efficient plants have evolved 3 major mechanisms:

- (i) increasing the surface and density of the roots resulting in more exploration of the soil volume (Vance et al., 2003),
- (ii) the rhizosphere acidification by root exudates (Neumann and Romheld, 1999) and H⁺ efflux (Tang et al., 2004; Shen et al., 2006; Zhou et al., 2009), and
- (iii) enhancing P utilization (internal mechanisms associated with conservable use of absorbed P at the cellular level) (Raghothama, 1999; Bates and Lynch, 2001; Vance et al., 2003).

Both intracellular and secreted acid phosphatases are induced in higher plants in response to P starvation (Duff et al., 1994). Intracellular acid phosphatases are likely to play a role in internal P homeostasis, whereas secreted acid phosphatases may be pertinent to external P acquisition (Duff et al., 1994). Phytate (myo-inositolhexakisphosphate; InsP₆) is a major storage form of P in seeds and pollens. Hydrolytic enzymes that catalyze the breakdown of phosphomonoester bonds in InsP₆ are collectively known as phytases. A number of phytase-encoding genes have been identified in the plant kingdom (Hegeman and Grabau, 2001; Rasmussen et al., 2003; Xiao et al., 2005). By sequence homology, currently known plant phytases are classified into 2 families:

histidine acid phosphatases and purple acid phosphatases, which were first discovered in maize (Maugenest et al., 1997) and soybean (Hegeman and Grabau, 2001), respectively. Both maize and soybean phytases were temporally expressed at the early stages of germination, which suggests their role in the mobilization of stored InsP₆-P to sustain the seedling growth. However, the physiological role of plant phytases in external P acquisition is still poorly understood. Although Pi deficiency elicits phytase activity in plant roots (Li et al., 1997; Hayes et al., 1999), extracellular phytase activities have been detected in several plant species (Richardson et al., 2000). Previous studies indicated that P starvation of tobacco seedlings triggered a substantial increase in the specific phytase activity in root exudates (Lung and Lim, 2006; Lung et al., 2008). Therefore, it could be assumed that an effective way to increase P-efficiency is to develop P-efficient cultivars, which can achieve a high yield under P deficiency condition.

Common bean varieties vary in their adaptation to P limitation (Vadez and Drevon, 2001). In order to investigate to what extent this variation may be related to maintain oxygen consumption, proton efflux and phytase activity by nodulated roots, 2 bean genotypes (115 and 147) were grown in hydroaeronic culture in a glasshouse at 2 levels of phosphorus supply corresponding to P sufficiency or P limiting.

MATERIALS AND METHODS

Hydroaeronic culture

The experiment was carried out in a temperature controlled greenhouse in hydroaeronic culture. The experiment concerned 2 common bean genotypes (The recombinant inbred lines 115 and 147 originating from the crosses between BAT477 and DOR364) and 2 levels of P supply (15 and 250 μmol P plant⁻¹ week⁻¹) in the nutrient solution. Seeds were surface sterilised and pre-germinated in agar during 4 days. Thereafter, roots of uniform seedlings were placed during 30 min into liquid inoculant containing the strain CIAT 899 of *Rhizobium tropici*. Seedlings were transferred into plastic vats by gently passing them through the hole of a rubber stopper with cotton wool fixed at the hypocotyl level. Each vat corresponding to each P treatment held 20 plants and 20 l of nutrient solution (Vadez et al., 1996), containing 2 mM urea, 1.65 mM CaCl₂, 1.0 mM MgSO₄, 0.7 mM K₂SO₄, 8.5 μM Fe as sequestrene, 6 μM MnSO₄ · H₂O, 4 μM H₃BO₃, 1 μM ZnSO₄ · 7H₂O, 1 μM CuSO₄ · 5H₂O, 0.1 μM Na₂ MoO₄ · 2H₂O and each level of P supply as KH₂PO₄. Solution pH was kept constant at 6.8 by continuous titration with 0.1 M KOH using a pH stat system. At 18 days after transplanting (DAT), each plant was transferred to 1-l serum bottles, wrapped with aluminium foil to maintain darkness in the rooting environment and containing the same nutrient solution previously described but with 1 mM of urea. This nitrogen source, prevent nitrogen deficiency which would occur between the exhaustion of cotyledon storage and the establishment of symbiosis. It does not inhibit the nodulation in common bean (Vadez et al., 1999). The nutrient solution was weekly replaced, and the urea supply was interrupted at 25 DAT. The nutrient solution was permanently aerated by compressed airflow provided by plastic tubes into the vats and bottles. After the measurements of O₂ consumption and proton efflux, plants were harvested at 42

DAS. Shoot, root and nodule DW were determined after drying for 3 days at 70°C.

Phytase assays

Nodules, roots and cotyledons (0.1 g) were ground separately in a mortar with an extraction mixture consisting of 500 µl acetate-buffer (0.1 M pH 5), 6 mM β-mercaptoethanol, 0.1 mM phenyl methyl sulfonyl fluoride (PMSF) and 6 g insoluble polyvinylpyrrolidone (PVPP). The homogenate was centrifuged at 30 000 g at 4°C for 30 min. For phytase activity, 100 µl of nodule and cotyledons crude extract was incubated during 90 min at 37°C with a mixture of 200 µl of acetate-buffer and 200 µl of substrate (phytic acid 0.2% as corn sodium salt sigma CAS 14306-25-3). The reaction was stopped by the addition of 0.5 ml trichloroacetic acid 10% and the mixture was centrifuged at 13 000 g for 5 min. Another aliquot of 100 µl of nodule extract received 200 µl of acetate-buffer and 200 µl of the phytic acid substrate, but the reaction was stopped immediately without incubation and the mixture centrifuged. Concentration of Pi in the extracts was measured spectrophotometrically at 630 nm using malachite green (Ohno and Zibilske, 1991). The phytase activity was calculated as the difference between the Pi in the extracts with and without incubation and expressed in nmol of Pi released per min per g of nodule fresh mass.

Proton efflux measurements

To compensate the acidification of the nutrient solution during the hydroaerobic culture, the pH was measured daily. 50 ml aliquot of the nutrient solution was taken from each 1 l bottle and corrected to pH of 6.8, if necessary, by adding an automatic titrator (Methrom) of a measured quantity Q (mol) of a 0.1 M KOH solution, according to the following formula:

$$Q = C \cdot V \cdot 10^{-3}$$

with C, concentration of the solution in M; V, volume of solution used in mL.

Oxygen exchange measurements

The *in situ* measurement of the oxygen uptake by the nodulated-roots was measured with an oxymeter (Abiss, Villemoisson, France) at 35 - 42 days after sowing (DAS) in the glasshouse as previously described by Jebara and Drevon (2001). The circulation of the gaseous phase in the circuit from the nodulated-root environment through the oxymeter was driven by a peristaltic pump with a flow of 400 ml.min⁻¹. The response of nodulated-root respiration (Conr) to external pO₂ was assayed during 20 min periods successively at 21, 25, 30 and 40 KPa O₂. Between each confinement, the circuit was opened and swept by a renewed gaseous mixture monitored by mass-flowmeters (Tylan, la Verpillère, France). The nodulated-root respiration (Conr) was calculated as µmol O₂ consumed h⁻¹ plant⁻¹ as [(initial-final)pO₂] [V/24,2] [60/t] with t (min), duration between initial and final O₂ measurements; V (l), volume of 1 mol pure gas in the experimental conditions.

Statistical analysis

The analysis of variance and the standard deviation of the means were performed with the statistica software to determine the significance (at P < 0.05) of differences in biomass data for symbiotic effectiveness. The statistical analysis of the regression model of

nodulated-root respiration as a function of external O₂ concentration was performed with covariance analysis. If the correlation between nodulated roots O₂ consumption and O₂ concentration was significant, the mean values and standard deviation of the model parameters were calculated. For each parameter, values (means of 6 replicates ± SD) followed by the same letters are not significantly different at 5% according to Fisher's LSD test.

RESULTS

Plant growth

Under P sufficiency (250 P), bean lines expressed a similar and higher potentially of plant growth (Figure 1A). P limiting (15 P) decreased significantly this parameter in the 2 lines, but this effect was more pronounced in 147 than in 115 (- 70 and - 58% as compared to the control, respectively). The nodule biomass (Figure 1B) which was higher for 115 than for 147 at 250 P, decreased significantly under 15 P, though to a higher extent for 147 (- 95%) than for 115 (- 85%). The P deficiency decreased lesser the nodule number (Figure 1C) than the nodule mass in the 2 lines. In addition, nodulation was more sensitive to P limiting than the plant growth.

Symbiotic nitrogen fixation and total phosphorus content

Under P limiting, both lines displayed significantly higher P concentration in nodules as compared to shoots and roots (Figures 2A, 2B and 2C). Similarly to the plant growth, P shortage restricted significantly the plant P status, 147 being however more affected than 115. In the former line, the reduction in P content ranged from - 70% for shoots and nodules to - 90% in roots, while it varied between - 65% for shoots and nodules to - 85% for roots in 115. The quantity of nitrogen fixed by plant (Figure 2D) was higher in the 2 lines under optimal P supply (250 P). P shortage led to a significant reduction of the symbiotic nitrogen fixation in both lines, this negative impact being however more pronounced in 147 than in 115 (- 75 and - 65%, respectively). In order to assess the variability in phosphorus use efficiency for symbiotic nitrogen fixation in bean, the ratio of N fixed per plant and nodule total P content was calculated. This parameter has previously been shown to vary with P supply (Vadez et al., 1999). Under P limiting conditions, this ratio was higher for 115 than for 147 (37 and 31 N fixed mmol plant⁻¹ / Total P mmol g⁻¹ nodules DW, respectively).

Nodulated root respiration

Nodulated root oxygen consumption (Conr) was O₂- dependent (Figure 3). Indeed, in both genotypes, the external pO₂ induced a significant increase in Conr regardless of P supply. Unlike 147 line, the Conr was significantly higher at 15 P than at 250 P for 115. At 15 P, the Conr

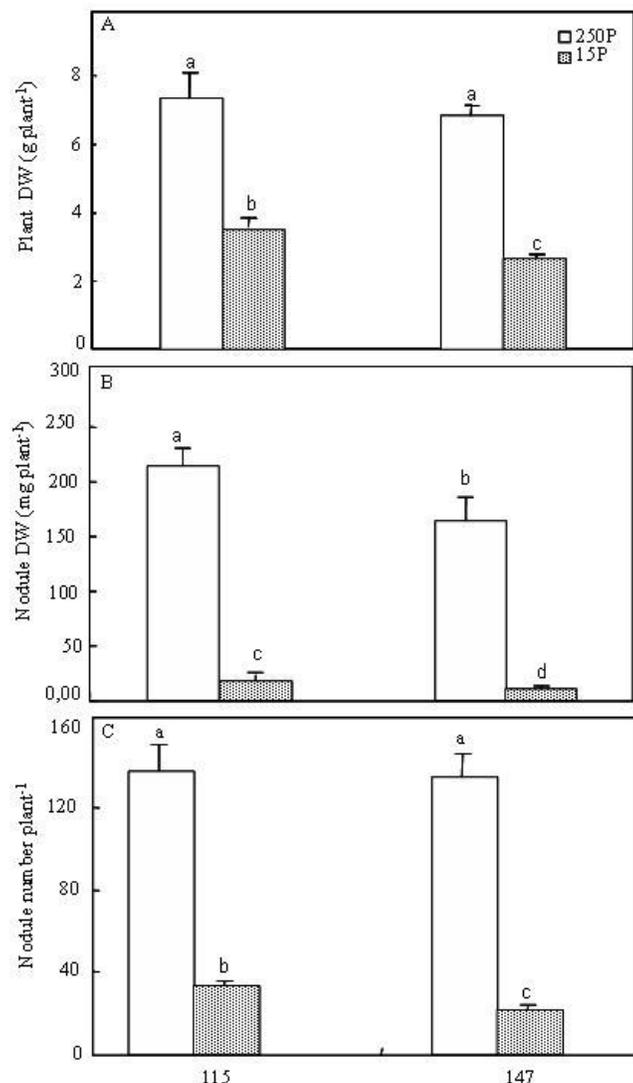


Figure 1. Effect of P supply on plant growth (A), nodule biomass (B) and nodule number (C) in common bean genotypes 115 and 147. For each parameter, values (means of 6 replicates \pm SD) followed by the same letters are not significantly different at 5% according to Fisher's LSD test.

increased significantly in 115 for external pO_2 above 25 KPa. The critical oxygen pressure ($COP=pO_2$ under which the respiration is limited by a lack of O_2) could be calculated as the first derivative of the curvilinear regression of $Conr$ as a function of pO_2 . For the 2 lines, the COP varied from 30 KPa O_2 at 250P. At 15P, the COP was even higher than these values since a linear response of $Conr$ as a function of pO_2 was observed in the range of the explored pO_2 .

The nodule conductance, defined as the nodule permeability per unit nodule area (Figure 4A) could be calculated by dividing the slope of the linear part of the regression of $Conr$ as a function of external O_2 concentrations (21, 25, 30 KPa) by the nodule area. This parameter

(Figure 4B) was similar in both lines at 250 P. Under 15 P, the nodule conductance increased significantly by 2.2 folds in 115 and 5.3 folds in 147.

Proton efflux

Whether daily measured or cumulated, the proton efflux in 115 was significantly greater under P deficiency than under P sufficiency (Figure 5). For instance, the cumulated H^+ efflux for 115 was 25% higher at 15 P than at 250 P, whereas for 147 this parameter increased slightly at 250 P. Under P limiting, the proton efflux cumulated or per unit of nodule-root biomass was respectively 20 and 25% greater for 115 than 147 (data not shown).

Phytase activity

The nodule phytase activity increased significantly in the 2 lines at 15P, but this effect was more pronounced in 115 than 147 (respectively 1.4 and 1.1 nmoles $Pi\ h^{-1}\ g^{-1}$ nodule FW) (Figure 6). In both lines, the phytase activity was higher in roots than in nodules. Despite P limiting increased significantly this parameter, 115 maintained higher values as compared to 147. The phytase activity measured in cotyledons was higher than in roots and nodules. In addition, this parameter was higher in 115 (12 nmoles $Pi\ h^{-1}\ g^{-1}$ nodule FW) than in 147 (9 nmoles $Pi\ h^{-1}\ g^{-1}$ nodule FW).

DISCUSSION

In this work, the growth of bean plants, estimated by the quantity of biomass production, was higher in the 2 lines at 250 P. P limiting (15 P) adversely affected the plant growth, especially in 147. In the same way, 15 P severely reduced essential processes such as the nodule growth, nodulation and symbiotic nitrogen fixation. Yet, the plant response was genotype-dependent, since this negative effect was more pronounced in 147. Under P limiting, the greater P requirement for nodules than for shoot or roots especially in 115 (Figure 2A, 2B and 2C) agrees with the previous studies reporting that the P content is threefold larger in nodules than in shoots for *Phaseolus vulgaris* (Araujo and Teixeira, 2000; Kouas et al., 2005), *Acacia mangium* (Ribet and Drevon, 1996) and *Glycine max* (Ribet and Drevon, 1995). The high requirement of P for nodules might be related to high energy requirement of the symbiotic nitrogen fixation process (Israel, 1987), that is, the equivalent of 30 ATP per N_2 reduced (Salsac et al., 1984). Interestingly we found higher phytase activity in cotyledons especially in 115. Phytate (myoinositolhexakisphosphate) is a major storage form of phosphorus (P) in seeds and pollens. It would be acceptable that at the early stage of development, the remobilisation of P in seeds by phytase facilitates the establishment of symbio-

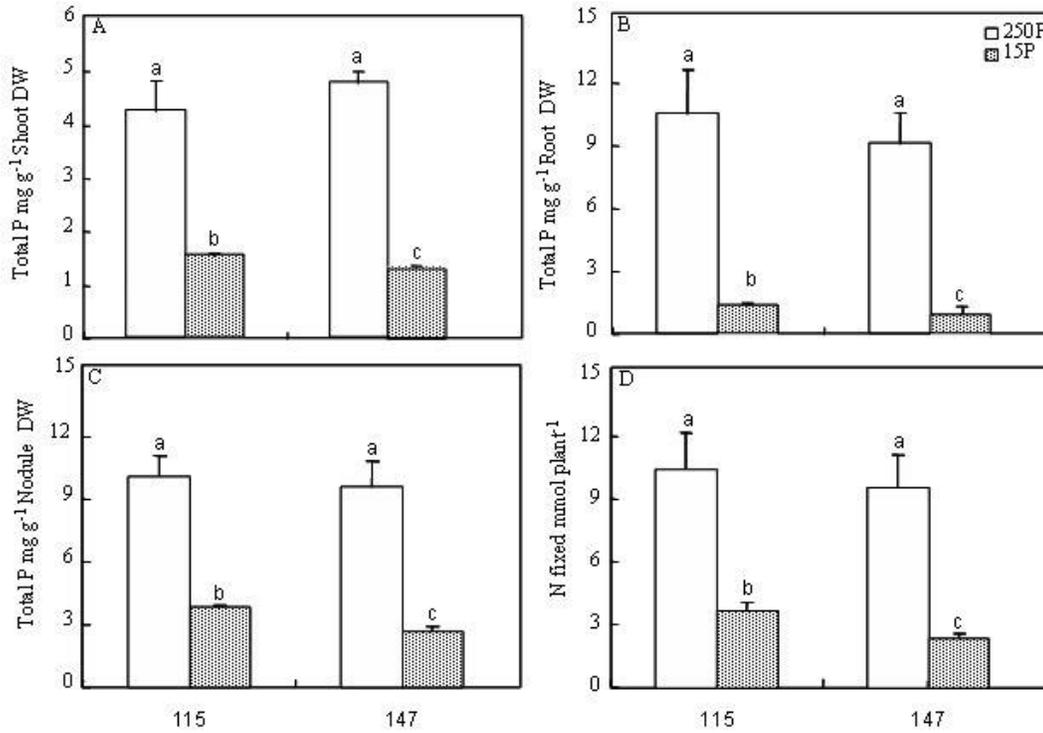


Figure 2. Effect of P supply on shoots (A), roots (B), nodules (C) total P content and symbiotic nitrogen fixation (D) in common bean genotypes 115 and 147. For each parameter, values (means of 6 replicates ± SD) followed by the same letters are not significantly different at 5% according to Fisher's LSD test.

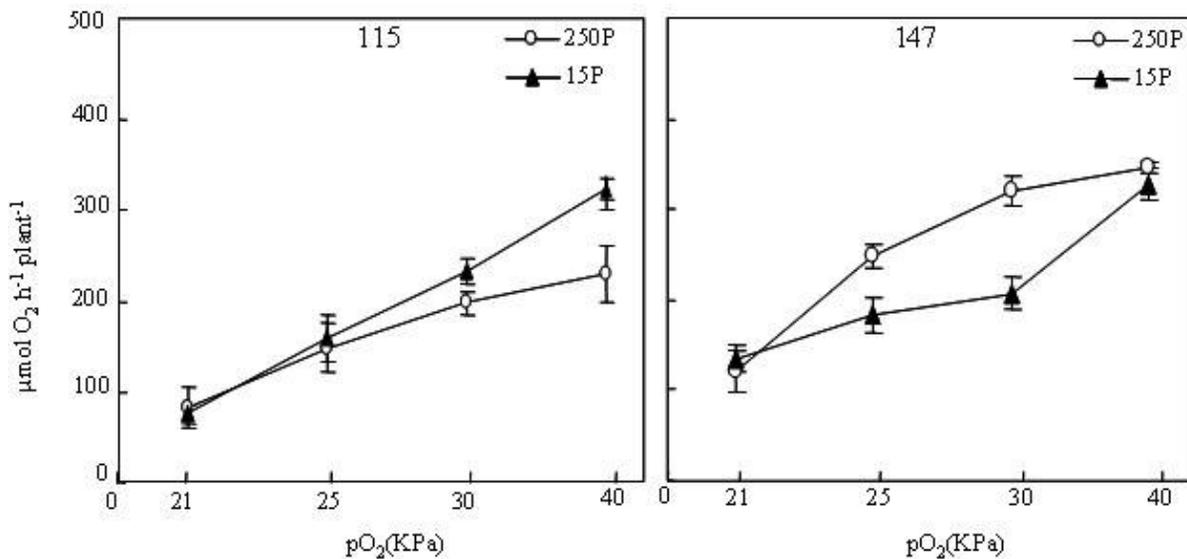


Figure 3. Effect of P supply on cumulated proton efflux of common bean genotypes 115 and 147. For each parameter, values (means of 6 replicates ± SD) followed by the same letters are not significantly different at 5% according to Fisher's LSD test.

sis suggesting that the infection and nodule initiation is not limited by P deficiency (Valverde and Wall, 1999).

Moreover, it seems that the accumulation of P in seeds

constitutes an adaptive mechanism to ensure the initiation of growth of the seedlings and the establishment of symbiosis (Valverde et al., 2002). In this work, the

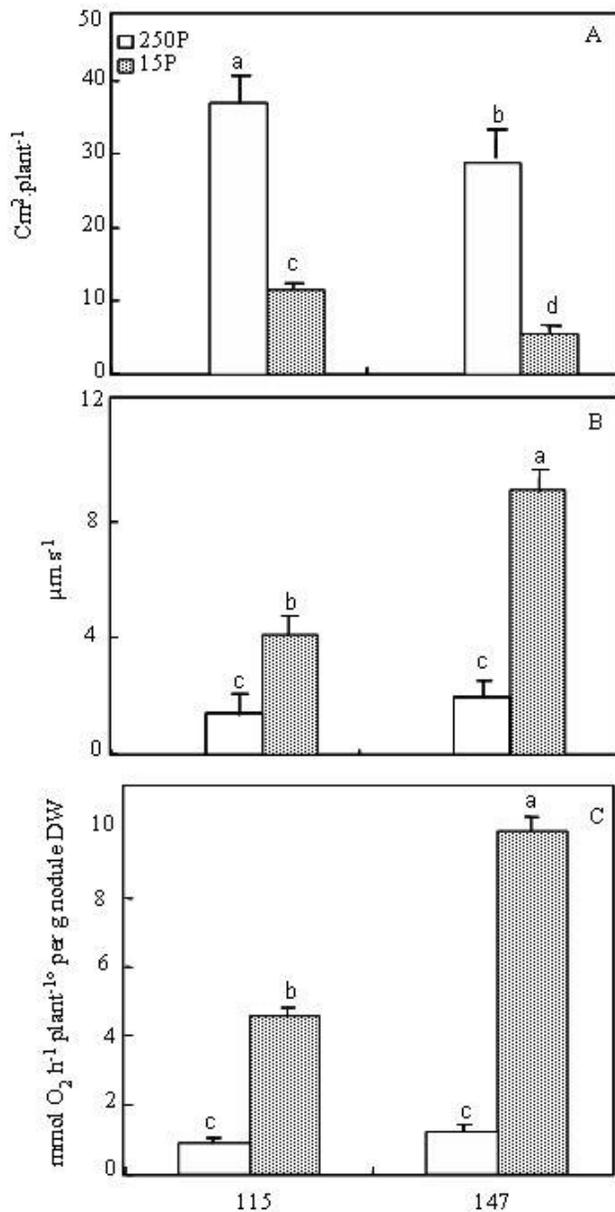


Figure 4. Effect of Rhizosphere pO₂ on *in situ* measurements of common bean nodulated-root O₂ consumption of bean genotypes 115 and 147 when supplied with 15P and 250P. For each parameter, values (means of 6 replicates ± SD) followed by the same letters are not significantly different at 5% according to Fisher's LSD test.

tolerance of 115 line was associated to a higher phytase activity in cotyledons and a better nodulation under P limiting. Both maize and soybean phytases were temporally expressed at the early stage of germination, which suggests their role in the mobilization of stored InsP₆-P to nourish seedling growth (Maugenest et al., 1997; Hegeman and Grabau, 2001). Our results showed a significant increase of phytase activity in roots and nodules in bean, with 115 maintaining a higher value. Although Pi deficiency elicits phytase activity in plant

roots (Hayes et al., 1999; Li et al., 1997a, b) and extracellular phytase activities have been detected in several plant species (Richardson et al., 2004). Previous studies indicated that P starvation of tobacco seedlings triggered a substantial increase in the specific phytase activity in root exudates (Lung and Lim, 2006; Lung et al., 2008). In this work, nodule phytase activity is likely to be derived from plant cells sight that a higher activity of this enzyme was observed in roots and was increased under P limiting. Phytic acid seems to play important roles in cellular metabolism, such as phosphate storage and retrieval in plant tissues, as major metabolic pool in inositol phosphate and pyrophosphate pathways, in energy currency and in ATP regeneration, in RNA export and DNA repair and as an anti-oxidant (Raboy, 2003). Appreciable amounts of phytate were detected in root and crown tissues of alfalfa (Campbell et al., 1991) and phytate was identified as the putative substrate for phytase in maize roots (Hübel and Beck, 1996). The induction of a higher phytase activity under P deficiency, as observed in bean roots and nodules (Figure 6), may enhance the P utilization from phytate in the plant and contribute to improve its adaptation to low P soils.

In this work, the proton release by nodulated-roots was higher in the 115 (most tolerant genotype to P deficiency) (Figure 5) under N nutrition, depending upon the rhizobial symbiosis. The better behaviour of 115 compared to 147, considering the biomass production, confirms other works documenting the relatively good adaptation of bean to P deficiency (Tang et al., 2003). The higher proton efflux in 115 correlated with its better adaptability to P limiting, suggesting an involvement of the root acidification capacity in the adaptation of common bean to this abiotic constraint. Sas et al. (2003) reported that H⁺ extrusion in P-deficient plants were, on average, 2 to 3-fold greater than organic acid exudation. In addition, the excess of cation content in plants was higher at low P supply (1 mmol P m⁻³) but decreased with increasing P supply. Similarly, Neumann and Romheld (1999) documented that phosphorus deficiency increased the proton efflux in roots of tomato and white lupin. Recently, Zhou et al. (2009) showed that faba bean can release significant amounts of proton, in comparison with soybean and maize. This result could partly explain why faba bean utilizes sparingly soluble P more effectively than soybean and maize and is of high significance in identifying the mechanisms behind interspecific facilitation of P uptake by intercropped species, especially when grown on calcareous soils. For 147, the proton efflux was slightly higher at 250 P as compared to 15 P, suggesting that part of the proton efflux does relate to symbiotic nitrogen fixation. According to Hinsinger et al. (2003), nitrogen forms affect rhizosphere acidification by influencing the balance of anion and cation uptake by plants. In the same way, legumes, relying on N₂ fixation, generally take up more cations than anions and thus extrude into the rhizosphere proportionally more H⁺ than OH⁻ to balance the charges (Tang et al., 1997).

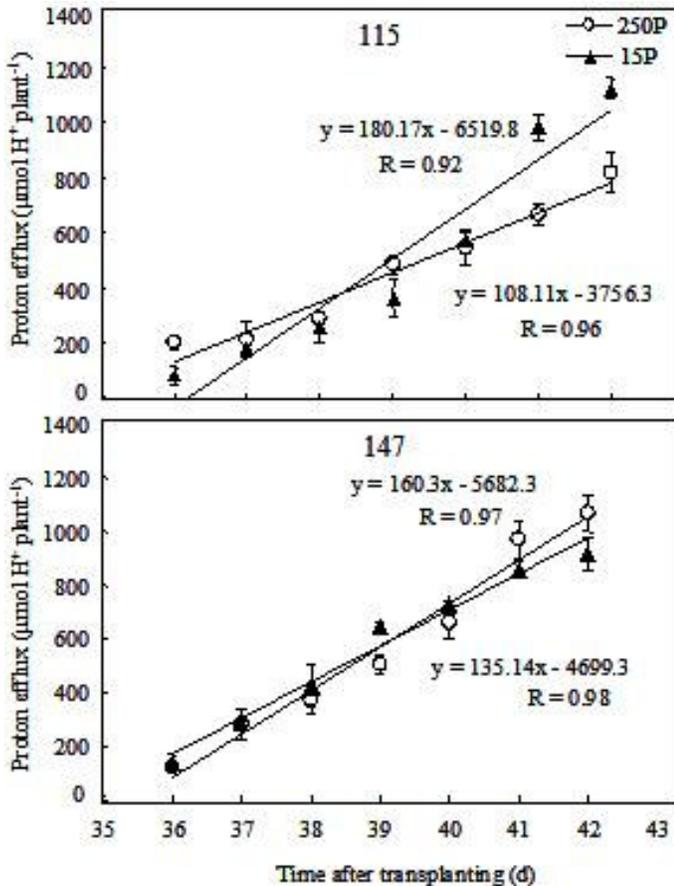


Figure 5. Effect of P supply on nodule area (A), nodule conductance (B) and nodulated-root O_2 consumption per g nodule DW (C) of common bean genotypes 115 and 147. For each parameter, values (means of 6 replicates \pm SD) followed by the same letters are not significantly different at 5% according to Fisher's LSD test.

The nodulated-roots oxygen consumption in the 2 lines increased with the external pO_2 , indicating that the nodule metabolism is limited because of lacking oxygen. This result can be explained by the existence of a physical limitation to the oxygen diffusion. Witty et al. (1987) and Masepohl et al. (1993) showed respectively in pea and lupin that the O_2 concentration decreased quickly with the penetration of a microelectrode specific to O_2 in the internal cortex, from where the role which would play the internal cortex in the conserve of the conditions necessary to maintained nitrogenase activity. Generally, the variation of oxygen diffusion would be dependent on cell deformation of external or internal nodule cortex (Serraj et al., 1995; Vadez et al., 1996). P limiting (15 P) increased significantly nodulated-root O_2 consumption per g nodule DW and nodule conductance, this effect being re marked for 147 than for 115 (Figures 4A and 4C). This increase of the oxygen diffusion under P limiting was previously observed in soybean (Ribet and Drevon, 1995)

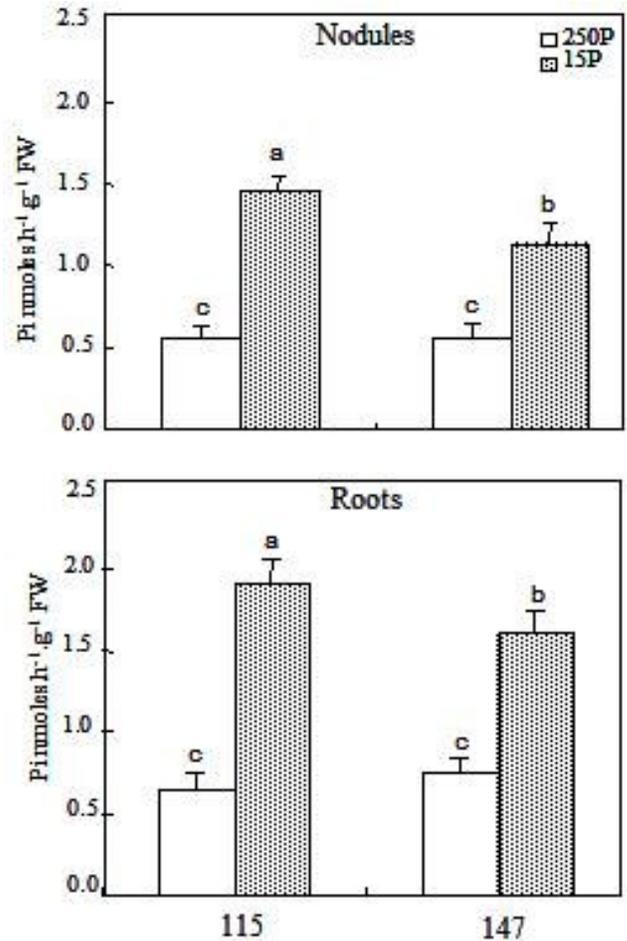


Figure 6. Effect of P supply on nodules and roots phytase activities of common bean genotypes 115 and 147. For each parameter, values (means of 6 replicates \pm SD) followed by the same letters are not significantly different at 5% according to Fisher's LSD test.

and in common bean (Vadez et al., 1996). According to Vadez et al. (1996), increased nodule conductance to O_2 under P deficiency might result from:

- (i) The enhanced O_2 limitation following the activation of the wasteful O_2 alternative respiration in nodules
- (ii) The direct effect of this nutrient constraint on nodule O_2 conductance which induces alternative respiratory pathways so that harmful excess oxygen can be scavenged.

These last authors demonstrated a genotypic variation concerning the nodule conductance under P limiting, suggesting a relation between the tolerance of the N_2 fixing plants to P deficiency and the nodule conductance. Our results confirm this relation between the respiration linked to nitrogenase activity and P availability in bean. Two competing hypotheses have been proposed to explain the control of dinitrogen fixation in legumes:

according to Minchin et al. (1985), Hunt and Layzell (1993) and Witty and Minchin (1998), the variable oxygen barrier in the outer cortex of legume nodules regulates the diffusion of oxygen into the infected tissues and thus controls respiration and dinitrogen fixation. Alternatively, the regulation of dinitrogen fixation is explained in terms of a feedback control (Schubert, 1995; Schulze, 2003). So, the feed back regulation is the primary mechanism, which regulates dinitrogen fixation according to host plant demand (Schubert, 2007).

In conclusion, the key traits of the tolerant line (115) to P limiting were:

- (i) A higher phytase activity in cotyledons roots and nodules to remobilise phosphorus, hence facilitating the symbiosis establishment, nodulation, which increases the phosphorus use efficiency for symbiotic nitrogen fixation,
- (ii) A higher proton efflux to acidify the medium and increased phosphorus uptake, and
- (iii) The ability to regulate the oxygen consumption by nodulated-roots and maintained nitrogenase activity.

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